CRY TOXINS FROM *BT* BOLLGARD II® COTTON SEEDS: AN AFFORDABLE SOURCE FOR INSECT BIOASSAYS

Suresh R Jambagi[1,2*], M Mohan[2], Upasna Selvapandian[2], K Muralimohan[1] and T Venkatesan[2]

[1]Department of Entomology, University of Agricultural Sciences, Bangalore 560065, Karnataka, India
[2]Division of Genomic Resources, ICAR-National Bureau of Agricultural Insect Resources, Bengaluru 560024, Karnataka, India
*Email: jambagisuru@gmail.com (corresponding author): ORCID ID 0000-0002-4678-8396

ABSTRACT

The relative expression of *Bacillus thuringiensis* (*Bt*) Cry1Ac and Cry2Ab toxins from seeds of *Bt* Bollgard II® cotton was quantified using Enzyme Linked Immunosorbent Assays (ELISA) method. The ELISA technique involves binding a sample or calibrator to a solid-phase antibody, forming a "sandwich complex" with an enzyme-labeled antibody. The resulting enzyme substrate reaction produces a product proportional to the antigen concentration. The study reveals that each gram of *Bt* Bollgard II® seed powder contains 3.25 µg Cry1Ac and 182.32 µg Cry2Ab, with Cry2Ab expression 56 times higher. Variability in Cry protein expression is influenced by factors like temperature, genetic background, and plant maturity. The research emphasizes the importance of *Bt* plants, particularly *Bt* Bollgard II® seed powder as a valuable source for studying insect responses to combined Cry1Ac + Cry2Ab expression in bioassay studies.

Key words: ELISA, crystal toxins, *Bacillus thuringiensis*, Bollgard II®, bioassays, Cry protein expression, Cry toxin source, Cry1Ac, Cry2Ab, *Bt* cotton, insect pests

Insect pests pose a significant threat to global agricultural production, causing substantial economic losses annually (Kranthi et al., 2005a). To address this challenge, genetically modified (GM) crops expressing insecticidal proteins from the bacterium, *Bacillus thuringiensis* (*Bt*) have been widely adopted. These Cry proteins target specific insect pests in the gut, disrupting their feeding and ultimately leading to mortality (Tabone et al., 2014). *Bt* Bollgard II® cotton is one of the most widely used GM crops, expressing two Cry proteins, Cry1Ac and Cry2Ab for enhanced protection against bollworm pests of cotton, specifically the pink bollworm, *Pectinophora gossypiella* Saunders. Monitoring and managing the evolution of insect resistance to *Bt* toxins is crucial for sustaining the long-term efficacy of this technology. Bioassays, which assess the toxicity of *Bt* toxins on target insects, play a vital role in this process. Traditionally, *Bt* bioassays have relied on commercially available *Bt* formulations or purified Cry proteins from bacteria. However, these methods can be expensive, inconsistent, and time-consuming (Zhao et al., 2012). Meanwhile, more than 99.99% of the cotton growing area in India is covered with double gene *Bt* hybrids (*cry1Ac* + *cry2Ab*) and conducting resistance monitoring studies to dual toxins is a difficult task. So far, most of the *Bt* resistance monitoring studies depend on commercially available or purified Cry toxins, those for which the assay has to be conducted individually for each toxin. As *Bt* Bollgard II® cotton seeds having both Cry1Ac and Cry2Ab toxins, the seed powder can be used as a toxin source upon quantification of toxin present in it.

The present investigation deals with the direct extraction and quantification of Cry proteins from *Bt* Bollgard II® cotton seeds using Enzyme-Linked Immunosorbent Assay (ELISA). ELISA is a sensitive and specific immunological technique that utilizes antibodies to detect and quantify target proteins (Tabone et al., 2014). Compared to conventional methods, extracting Cry proteins from cotton seeds offers several advantages, including its cost-effectiveness, most convenient and high consistency (Yang et al., 2015). Therefore, the present investigation optimized the Cry toxin extraction procedure from *Bt* cotton seeds and employed ELISA technique for quantifying Cry1Ac and Cry2Ab toxins. Such quantification is essential for *Bt* resistance monitoring in target pest species. By providing a readily available and affordable source of Cry proteins for bioassays, one can contribute to the sustainable use of *Bt* technology in pest control and ensure the continued productivity of *Bt* cotton and other GM crops.

MATERIALS AND METHODS

Assays on quantification of Cry toxins were

conducted at Division of Genomic Resources, ICAR-National Bureau of Agricultural Insect Resources, Bengaluru (13.0270° N, 77.5843° E), Karnataka, India during 2021-22. The seeds of Bollgard II® *Bt* cotton hybrid, MRC 7918 BG-II (Mahyco Pvt. Ltd.) and non-*Bt* cotton hybrid, SCS-793 were used for protein estimation experiment. 500 grams of each *Bt* and non-*Bt* seeds were dipped in 0.40% sodium hypochlorite (NaOCl) for 10 min to sterilize the seed surface. Later, the seeds were thoroughly washed in distilled water. The shade dried seeds were decorticated and ground to a fine powder in a motorized electric grinder (Johnson® Kitchen Mate) to produce fine seed powder. To get homogenized product, the seed powder was passed through 250µM sieve. The resultant *Bt* and non-*Bt* seed powder was stored at room temperature (27°C) and used further for the quantification of Cry1Ac and Cry2Ab protein through Enzyme-Linked Immunosorbent Assay (ELISA) using DesiGen™ Quan-T ELISA 96-well plate kit (DesiGen, Jalna, India).

Estimation of Cry1Ac and Cry2Ab protein was done independently. Five milligrams of homogenized *Bt* cotton seed powder was taken in 1.5 mL micro centrifuge tube (Spinwin™ Micro Centrifuge Tube) and 500 µL of ice-cold 1X extraction buffer was added. The sample was further macerated with micropestle for 30-60 seconds and kept it in mini cooler for 10 min. Then the sample was centrifuged at 8000 rpm for 15 min at 4°C. Later, the supernatant (sample extract) was carefully collected and trypsinization was done only for Cry1Ac estimation by adding 5.25 µL of trypsin and 3.75 µL of 50 mM Phenylmethanesulfonyl Fluoride (PMSF) to 150 µL of seed sample extract. Further, the sample dilution (1:4 for Cry1Ac; 1:20 for Cry2Ab) was done by using 1X diluent buffer. The positive and negative controls were prepared just for qualitative verification of the plate. Five milligrams of positive and negative control (supplied with kit) were weighed separately and added to 1.5 mL micro centrifuge tube. 500 µL of 1X wash buffer/ 1X buffer A was then added to both the control and macerated with micro pestle. To obtain the supernatant, both the controls were centrifuged at 8000 rpm for 30 sec at 4°C. The collected supernatant was further stored in -80 °C until further use. Meanwhile, the dilution of positive control (1:4) was done using 1X diluent buffer. 100 µL of both the controls were used in the ELISA assay per well. Working stock solutions of 20 ng/mL Cry1Ac and 160 ng/mL Cry2Ab were prepared using the Cry1Ac (1 µL/ mL) and Cry2Ab protein stocks (16 µL/mL) provided with kit in 1X diluent buffer. The different Cry1Ac protein standards viz., 0.625, 1.25, 2.50, 5.00, 10.00, 20.00 ng/ mL and Cry2Ab protein standards viz., 2.50, 5.00, 10.00, 20.00, 40.00 and 80.00 ng/mL were generated using the prepared working stock solutions. The secondary antibodies (Ab2) for Cry1Ac and Cry2Ab were diluted in 1X diluent buffer at a dilution factor of 1:1000. Further, 150 µL of prepared Ab2 was added to all the well of ELISA plate. Later, 100 µL of different standards, blank (1X diluent buffer), positive control, negative control and unknown samples were added to respective wells. The ELISA plate was further incubated at 37°C for 1.50 h in humid environment (incubator). After incubation, the samples were discarded and ELISA plate was washed twice with 1X wash buffer and patted thoroughly on tissue paper to get it completely dried. For protein detection, tertiary antibody (Ab3) provided with the kit was diluted in 1X diluent buffer to attain the dilution factor of 1: 1000. 250 µL of prepared conjugate was added to all the ELISA plate wells, then mixed and incubated at 37°C for 45 min. Later, the content was discarded and plate was washed twice with 1X wash buffer and patted on tissue paper for ensuring proper drying.

In each well of ELISA plate, 250 µL of freshly prepared 1 mg/ mL pNPP solution with 1X substrate buffer was added. After this, the plate was incubated in the dark at room temperature (27 °C) for 30 min (for Cry1Ac) to 40 min (for Cry2Ab). Later, the absorbance of the ELISA plate was measured at 405 nm using a Biorad iMark microplate absorbance reader. The standard curve was prepared using Sigma Plot® version 8.01 (4-parameter sigmoidal curve fit) based on the Optical Density (OD) values (average of triplicate samples) plotted against different concentrations (Standards) of Cry1Ac and Cry2Ab proteins. The Cry1Ac and Cry2Ab toxin content of the sample solution was extrapolated using the linear regression equation of the standard curve and the total protein was estimated for the unknown samples. Linear regression: y= a + bx, where y= Absorbance (OD value) of the sample; a= Intercept; b= Slope and; x= Concentration of Cry1Ac or Cry2Ab as ng/mL in unknown sample. To express Cry1Ac and Cry2Ab toxin present in each gram of seed powder, the following formula was used:

$$\mu g/\ g \text{ dry weight of Cry1Ac or Cry2Ab} = \frac{[(\text{ng/ mL value from Sigma Plot}^{®} \text{ software}) (\text{Dilution factor}) (\text{T:B ratio})]}{1000}$$

Where, dilution factor = dilution of sample i.e. 4 for Cry1Ac and 20 for Cry2Ab; Tissue to Buffer ratio (T:

B) = 500/ 5= 100. Since, the trypsinization process produces a protein fragment of 0.5 times the mass of the full-length protein, the assay results were multiplied by two to produce a true full-length Cry1Ac protein concentration.

RESULTS AND DISCUSSION

The prospect of utilizing ELISA for the detection and quantification of crystal toxins from *B. thuringiensis* subspecies was first given by Wie et al. (1982). In this procedure, a component of the reaction is either nonspecifically adsorbed or covalently attached to the surface of a solid phase, typically a microtiter well. This binding serves to facilitate the separation between the reactants that are bound (Cry proteins) and those that are free-labeled. In the prevailing method of utilizing the ELISA technique, a portion of the sample (i.e. Cry protein-containing *Bt* seed powder) or a calibrator containing the specific antigen (Ag) to be measured is introduced and allowed to bind with a solid-phase antibody (Ab). Following a washing step, an enzyme-labeled antibody is introduced, forming a "sandwich complex" consisting of solid-phase Ab-Ag-Ab enzyme. Subsequently, unbound antibody is removed through washing, and an enzyme substrate is introduced. The resulting quantity of generated product is directly proportional to the concentration of the antigen present in the sample (Engvall, 2010; Aydin, 2015).

In the present work, the amount of Cry1Ac and Cry2Ab proteins present in the Bollgard II® *Bt* seed powder was quantified. From the ELISA assay and subsequent standard curve generation (Absorbance at 405 nm *vs* Cry protein concentration in ng/ mL), it was found that each gram of *Bt* Bollgard II® seed powder contained 3.25 µg Cry1Ac and 182.32 µg Cry2Ab toxic proteins (Fig. 1). The expression of Cry2Ab protein was 56 times greater than the Cry1Ac (Cry2Ab/ Cry1Ac= 56.10) as depicted in Fig. 1. Earlier, Dhurua and Gujar (2011) estimated that each milligram (mg) of *Bt* BG II seed powder contained 0.005 ng of Cry1Ac and 15.385 ng of Cry2Ab proteins. The expression of Cry2Ab was almost 2797 times higher than the Cry1Ac. In a study conducted by Knight et al. (2016) revealed that, the Cry1Ac protein concentration in field-grown Bollgard II® cotton ranged from 3.98 to 12.08 µg/ g and Cry2Ab content ranged between 300.6 to 953.3 µg/ g fresh weight of leaves. Their findings also indicated that within the range of concentrations expressed in Bollgard II® cotton, the two proteins exhibited roughly equal significance. Moreover, alterations in the Cry2Ab content exerted a more pronounced impact on the larval mortality of *Helicoverpa armigera* compared to Cry1Ac. The latter contributed consistently to a mortality rate of 40 to 45% for concentrations exceeding 3 µg/g of leaf. In a recent study, Sheela (2023) observed a range of expression of Cry toxins in different *Bt* BG II cotton hybrids (1.13 to 3.37 µg/g of raw seed powder).

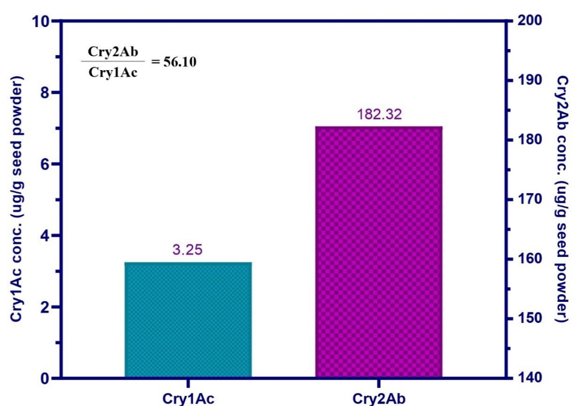

Fig. 1. Cry toxin content in *Bt* Bollgard II® cotton seed powder

The expression level of Cry proteins varies greatly due to various factors (Wan et al., 2005). Chen et al. (2005) noted that subjecting *Bt* transgenic cotton to high temperatures (37°C) significantly reduced the Cry1Ac protein content during the boll-setting stage. In certain conditions, the insecticidal protein levels in *Bt* cotton tissues may experience a substantial reduction, although the toxin level remains above the critical threshold (1.19 µg/ g as per Kranthi et al., 2005b), ensuring the maintenance of relatively high efficacy against insect pests. Moreover, the expression of the Cry1Ac gene in cotton lines is influenced by factors such as the site of gene insertion, gene construct, background genotype, epistasis, somaclonal mutations and the physical environment, with genetic background emerging as the most significant factor. Examination of Cry1Ac expression across a season in various cotton varieties indicated a reduction in protein levels as the plants matured (Poongothai et al., 2010).

The probit assays for different insects using the plant parts containing Cry toxins also have several advantages. So, *Bt* Bollgard II® seed powder specifically advantageous as a Cry toxin source in the bioassay studies, where the response of insect pest towards combined expression of Cry1Ac + Cry2Ab need to be studied (e.g. insect response to *Bt* Bollgard II® technology).

ACKNOWLEDGEMENTS

Authors thank Director, ICAR-National Bureau of

Agricultural Insect Resources, Bengaluru and Head, Department of Entomology, University of Agricultural Sciences, GKVK, Bengaluru for providing necessary infrastructure facilities. The first author gratefully acknowledges the award of the DST-INSPIRE fellowship (IF190948) by the Department of Science and Technology, Govt. of India.

AUTHOR CONTRIBUTION STATEMENT

SRJ- investigation, original draft preparation, methodology, software, data curation; MM-conceptualization, formal analysis, project administrator, supervision, draft reviewing and editing; US: formal analysis, visualisation; KM and TV- draft review and editing, formal analysis

FINANCIAL SUPPORT

No financial support.

CONFLICT OF INTEREST

No conflict of interest.

REFERENCES

Aydin S. 2015. A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. Peptides 72: 4-15.

Chen F, Wu G, Ge F, Parajulee M N, Shrestha R B. 2005. Effects of elevated CO_2 and transgenic *Bt* cotton on plant chemistry, performance, and feeding of an insect herbivore, the cotton bollworm. Entomologia Experimentalis et Applicata 115(2): 341-350.

Dhurua S, Gujar G T. 2011. Field-evolved resistance to Bt toxin Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), from India. Pest Management Science 67(8): 898-903.

Engvall E. 2010. The ELISA, enzyme-linked immunosorbent assay. Clinical Chemistry 56(2): 319-320.

Knight K, Head G, Rogers J. 2016. Relationships between Cry1Ac and Cry2Ab protein expression in field-grown Bollgard II® cotton and efficacy against *Helicoverpa armigera* and *Helicoverpa punctigera* (Lepidoptera: Noctuidae). Crop Protection 79: 150-158.

Kranthi K R, Dharmesh Y S, Sarmacharyulu Y. 2005a. Bollgard cotton in India: insect resistance management for sustainable use. Current Science 89(1): 70-78.

Kranthi K R, Naidu S, Dhawad C S, Tatwawadi A, Mate K, Patil E, Bharose A A, Behere G T, Wadaskar R M, Kranthi S. 2005b. Temporal and intra-plant variability of Cry1Ac expression in *Bt*-cotton and its influence on the survival of the cotton bollworm, *Helicoverpa armigera* (Hübner) (Noctuidae: Lepidoptera). Current Science, pp.291-298.

Poongothai S, Ilavarasan R, Karrunakaran C M. 2010. Cry1Ac levels and biochemical variations in *Bt* cotton as influenced by tissue maturity and senescence. Journal of Plant Breeding and Crop Science 2(5): 96-103.

Sheela N. 2023. Bio-efficacy of UASD *Bt*-Cotton Event No. 78 against bollworms complex in Comparison with commercially available events in India. Ph.D. Thesis, Univ. Agril. Sci. Dharwad, p.75.

Tabone T, Bravo A, Gomez C. 2014. A Review of Cry Protein Detection with Enzyme-Linked Immunosorbent Assays. Toxins 6(10): 3547-3586.

Wan P, Zhang Y, Wu K, Huang M. 2005. Seasonal expression profiles of insecticidal protein and control efficacy against *Helicoverpa armigera* for *Bt* cotton in the Yangtze River valley of China. Journal of economic entomology 98(1): 195-201.

Wie S I, Andrews R E, Hammock B D, Faust R M, Bulla L A. 1982. Enzyme-linked immunosorbent assays for detection and quantitation of the entomocidal parasporal crystalline protein of *Bacillus thuringiensis* subspp. *kurstaki* and *israelensis*. Applied and Environmental Microbiology 43(4): 891-894.

Yang Y, Guo Y, Zhang G. 2015. Quantification of Cry1Ac and Cry1F *Bacillus thuringiensis* Insecticidal Proteins in Selected Transgenic Cotton Plant Tissue Types. Journal of Agricultural and Food Chemistry 63(24): 5758-5766.

Zhao J, Liu H, Yang Y. 2012. Bt-cotton seed as a source of *Bacillus thuringiensis* insecticidal Cry1Ac toxin for bioassays to detect and monitor bollworm resistance to Bt-cotton. Journal of Insect Science 12(1): 102.

(Manuscript Received: January, 2024; Revised: February, 2024;
Accepted: April, 2024; Online Published: May, 2024)
Online First in www.entosocindia.org and indianentomology.org Ref. No. e24922